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(57) Abstract

Methods and compositions for treating diabetes mellitus in a patient in need thereof are provided. The methods include administering to a patient a composition providing a gastrin/CCK receptor ligand, e.g., a gastrin, and/or an epidermal growth factor (EGF) receptor ligand, e.g., TGF-α, in an amount sufficient to effect differentiation of pancreatic islet precursor cells to mature insulin-secreting cells. The composition can be administered systemically or expressed *in situ* by cells transgenically supplemented with one or both of a gastrin/CCK receptor ligand gene, e.g., a preprogastrin peptide precursor gene and an EGF receptor ligand gene, e.g., a TGF-α gene. The methods also include transplanting into a patient cultured pancreatic islets in which mature insulin-secreting beta cells are proliferated by exposure to a gastrin/CCK receptor ligand and an EGF receptor ligand.

TREATMENT FOR DIABETES

INTRODUCTION

Field of Invention

This invention relates to a method for treating diabetes mellitus in an individual in need thereof by administering to the individual a composition comprising a gastrin/CCK receptor ligand and/or an EGF receptor ligand which effectively promotes differentiation of pancreatic islet precursor cells to mature insulin-secreting cells. The method is exemplified by administration of gastrin and transforming growth factor alpha (TGF-α) either alone or in combination to normal streptozotocin (STZ) induced diabetic and genetically predisposed diabetic Zucker rats.

Background

Diabetes is one of the most common endocrine diseases across all age groups and populations. In addition to the clinical morbidity and mortality, the economic cost of diabetes is huge, exceeding $90 billion per year in the US alone, and the prevalence of diabetes is expected to increase more than two-fold by the year 2010.

There are two major forms of diabetes mellitus: insulin-dependent (Type 1) diabetes mellitus (IDDM) which accounts for 5 to 10% of all cases, and non-insulin-dependent (Type 2) diabetes mellitus (NIDDM) which comprises roughly 90% of cases. Type 2 diabetes is associated with increasing age however there is a trend of increasing numbers of young people diagnosed with NIDDM, so-called maturity onset diabetes of the young (MODY). In both Type 1 and Type 2 cases, there is a loss of insulin secretion, either through destruction of the β-cells in the pancreas or defective secretion or production of insulin. In NIDDM, patients typically begin therapy by following a regimen of an optimal diet, weight reduction and exercise. Drug therapy is initiated when these measures no longer provide adequate

Since differentiation of protodifferentiated precursors occurs during late fetal development of the pancreas, the factors regulating islet differentiation are likely to be expressed in the pancreas during this period. One of the genes expressed during islet development encodes the gastrointestinal peptide, gastrin. Although gastrin acts in the adult as a gastric hormone regulating acid secretion, the major site of gastrin expression in the fetus is the pancreatic islets. Brand and Fuller, *J. Biol Cnem.*, *263*:5341-5347 (1988). Expression of gastrin in the pancreatic islets is transient. It is confined to the period when protodifferentiated islet precursors form differentiated islets. Although the significance of pancreatic gastrin in islet development is unknown, some clinical observations suggest a rule for gastrin in this islet development as follows. For example, hypergastrinemia caused by gastrin-expressing islet cell tumors and atrophic gastritis is associated with nesidioblastosis similar to that seen in differentiating fetal islets. Sacchi, *et al.*, *Virchows Archiv B*, *48*:261-276 (1985); and Heitz *et al.*, *Diabetes*, *26*:632-642 (1977). Further, an abnormal persistence of pancreatic gastrin has been documented in a case of infantile nesidioblastosis. Hollande, *et al.*, *Gastroenterology*, *71*:251-262 (1976). However, in neither observation was a causal relationship established between the nesidioblastosis and gastrin stimulation.

It is therefore of interest to identify agents that stimulate islet cell regeneration which could be of value in the treatment of early IDDM and in the prevention of β-cell deficiency in NIDDM.

Citations of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

RELEVANT LITERATURE

Three growth factors are implicated in the development of the fetal pancreas, gastrin, transforming growth factor α (TGF-α) and epidermal growth factor (EGF) (Brand and Fuller, *J. Biol. Chem.* *263*:5341-5347). Transgenic mice over expressing TGF-α or gastrin alone did not demonstrate active islet cell growth, however mice expressing both transgenes displayed significantly increased islet cell mass (Wang *et al*, (1993) *J Clin Invest* *92*:1349-1356). Bouwens and Pipeleers (1998) *Diabetoligia* *41*:629-633 report that there is a high proportion of budding β-cells in the normal adult human pancreas and 15% of all β-cells were found as

beta cells can be grown in culture for a time sufficient to expand the population of β-cells prior to transplantation. The methods and compositions find use in treating patients with diabetes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is an image that shows numerous insulin staining cells in the metaplastic ducts from the TGF-α transgenic pancreas upon immunoperoxidase staining. Figure 1B is an image that shows that most ductular cells stained less intensely for insulin, while occasional ductular cells did stain with the same intensity of insulin staining as the adjacent islets.

Figure 2A schematically shows the structure of the chimeric insulin promoter-gastrin (INSGAS) transgene. Figure 2B illustrates that the radioimmunassay of pancreatic extracts from INSGAS transgenic mice shows high levels of gastrin immunoreactivity that exceed the gastrin content in the gastric antrum expressed from the endogenous murine gene. The INSGAS transgenic mice had high expression of gastrin in the postnatal pancreas.

Figure 3A is an image of the pancreatic histology of an INSGAS/TGF-α mouse used in the study reported by Example 3. The INSGAS/TGF-α pancreas had some areas of increased ductular complexes and slightly increased interstitial cellularity. The field shown here had the most severely abnormal histology in the five animals used. Figure 3B is an image of the pancreatic histology of a control mouse from Example 3. Figure 3C is an image of the pancreatic histology of a TGF-α mouse from Example 3. This field of a TGF-α mouse pancreas from the study reported in Example 3 was typical and showed the interstitial cellularity and fibrosis combined with florid ductular metaplasia that has been described by Jhappan, *et al*, *supra*.

Figure 4A is a histogram graphically illustrating point=counting morphometric data which confirmed that at 17 weeks the pancreas of the INSGAS/TGF-α mice had lower duct mass than the pancreas of the TGF-α mice based on the study reported in Example 3. Figure 4B is a histogram which graphically illustrates point=counting morphometric data which show that co-expression of gastrin and TGF-α in the INSGAS/TGF-α pancreas significantly increased the islet mass compared to the islet mass of the corresponding non-transgenic control mice. Further, TGF-α expression alone does not increase islet mass. These data are based on the studies illustrated in Example 3.

vivo or *in vivo* with one or more nucleic acid expression constructs in an expression vector which provides for expression of the desired receptor ligand(s) in the pancreatic islet precursor cells. As an example, the expression construct includes a coding sequence for a CCK receptor ligand, such as preprogastrin peptide precursor coding sequence which, following expression, is processed to gastrin or a coding sequence for an EGF receptor ligand such as TGF-α, together with transcriptional and translational regulatory regions which provide for expression in the pancreatic islet precursor cells. The transcriptional regulatory region can be constitutive or induced, for example by increasing intracellular glucose concentrations, such as a transcriptional regulatory region from an insulin gene. Transformation is carried out using any suitable expression vector, for example, an adenoviral expression vector. When the transformation is carried out *ex vivo*, the transformed cells are implanted in the diabetic patient, for example using a kidney capsule. Alternatively, pancreatic islet cells are treated *ex vivo* with a sufficient amount of a gastrin/CCK receptor ligand and/or an EGF receptor ligand to increase the number of pancreatic β cells in the islets prior to implantation into the diabetic patient. As required, the population of pancreatic β cells is expanded in culture prior to implantation by contacting them with the same receptor ligand(s).

The subject invention offers advantages over existing treatment regimens for diabetic patients. By providing a means to stimulate the adult pancreas to regenerate, not only is the need for traditional drug therapy (Type 2) or insulin therapy (Type 1 and Type 2) reduced or even eliminated, but the maintenance of normal blood glucose levels also may reduce some of the more debilitating complications of diabetes. Diabetic complications include those affecting the small blood vessels in the retina, kidney, and nerves, (microvascular complications), and those affecting the large blood vessels supplying the heart, brain, and lower limbs (mascrovascular complications). Diabetic microvascular complications are the leading cause of new blindness in people 20-74 years old, and account for 35% of all new cases of end-stage renal disease. Over 60% of diabetics are affected by neuropathy. Diabetes accounts for 50% of all non-traumatic amputations in the USA, primarily as a result of diabetic macrovascular disease, and diabetics have a death rate from coronary artery disease that is 2.5 times that of non-diabetics. Hyperglycemia is believed to initiate and accelerate progression of diabetic microvascular complications. Use of the various current treatment regimens cannot

gastrin/CCK receptor ligand and an EGF receptor ligand, preferably TGF-$_\alpha$, either alone or in combination to the individual.

Another embodiment comprises providing a gastrin/CCK receptor ligand and/or an EGF receptor ligand to pancreatic islet precursor cells of explanted pancreatic tissue of a mammal and reintroducing the pancreatic tissue so stimulated to the mammal.

In another, the invention comprises providing a gastrin/CCK receptor ligand and/or an EGF receptor ligand to pancreatic islet precursor cells of explanted pancreatic tissue from a mammal to expand the population of β cells.

In another embodiment gastrin/CCK receptor ligand stimulation is effected by expression of a chimeric insulin promoter-gastrin fusion gene construct transgenically introduced into such precursor cells. In another embodiment EGF receptor ligand stimulation is effected by expression of an EGF receptor ligand gene transgenically introduced into the mammal. The sequence of the EGF gene is provided in USPN 5,434,135.

In another embodiment stimulation by a gastrin/CCK receptor ligand and an EGF receptor ligand is effected by coexpression of (i) a preprogastrin peptide precursor gene and (ii) an EGF receptor ligand gene that have been stably introduced into the mammal.

In another aspect the invention relates to a method for effecting the differentiation of pancreatic islet precursor cells of a mammal by stimulating such cells with a combination of a gastrin/CCK receptor ligand and an EGF receptor ligand. In a preferred embodiment of this aspect, gastrin stimulation is effected by expression of a preprogastrin peptide precursor gene stably introduced into the mammal. The expression is under the control of the insulin promoter. EGF receptor ligand, e.g., TGF-$_\alpha$, stimulation is effected by expression of an EGF receptor ligand gene transgenically introduced into the mammal. In furtherance of the above, stimulation by a gastrin and a TGF-$_\alpha$ is preferably effected by co-expression of (i) a preprogastrin peptide precursor gene and (ii) an EGF receptor ligand introduced into the mammal. Appropriate promoters capable of directing transcription of the genes include both viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus (CMV) promoter (Boshart *et al* (1985) *Cell 41*:521-530), the SV40 promoter (Subramani *et al* (1981) *Mol. Cell. Biol. 1*:854-864) and the major late promoter from Adenovirus 2 (Kaufman and Sharp (1982) *Mol. Cell. Biol. 2*:1304-13199). Preferably,

virus, adeno-associated virus, retrovirus, lentivirus, and the like. *See* Blomer *et al* (1996) *Human Molecular Genetics* 5 Spec. No:1397-404; and Robbins *et al* (1998) *Trends in Biotechnology 16*:35-40. Adenovirus-mediated gene therapy has been used successfully to transiently correct the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *See* Zabner *et a.* (1993) *Cell* 75:207-216.

Another aspect of the invention is a non-human mammal or mammalian tissue, including cells, thereof capable of expressing a stably integrated gene which encodes preprogastrin. Another embodiment of this aspect is a non-human mammal capable of coexpressing (i) a preprogastrin peptide precursor gene; and/or (ii) an EGF receptor ligand, e.g., a TGF-α gene that has been stably integrated into the non-human mammal, mammalian tissue or cells. The mammalian tissue or cells can be human tissue or cells.

Therapeutic Administration and Compositions

Modes of administration include but are not limited to transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds can be administered by any convenient route, for example by infusion or bolus injection by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and can be administered together with other biologically active agents. Administration is preferably systemic.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration. Pharmaceutically acceptable carriers and formulations for use in the present invention are found in Remington's *Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985), which is incorporated herein by reference. For a brief review of methods for drug delivery, *see* Langer (1990) *Science 249*:1527-1533, which is incorporated herein by reference.

In preparing pharmaceutical compositions of the present invention, it may be desirable to modify the compositions of the present invention to alter their pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, see Remingtons's

derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium and other divalent cations, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the therapeutic of the invention which is effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. The precise dose to be employed in the formulation also will depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective dosages can be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Suppositories generally contain active ingredient in the range of 0.5% to 10% weight; oral formulations preferably contain 10% to 95% active ingredient.

In the gene therapy methods of the invention, transfection *in vivo* is obtained by introducing a therapeutic transcription or expression vector into the mammalian host, either as naked DNA, complexed to lipid carriers, particularly cationic lipid carriers, or inserted into a viral vector, for example a recombinant adenovirus. The introduction into the mammalian host can be by any of several routes, including intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intranasally, intramuscularly, topically, transdermally, application to any mucous membrane surface, corneal installation, etc. Of particular interest is the introduction of the therapeutic expression vector into a circulating bodily fluid or into a body orifice or cavity. Thus, intravenous administration and intrathecal administration are of particular interest since the vector may be widely disseminated following such routes of administration, and aerosol administration finds use with introduction into a body orifice or cavity. Particular cells and tissues can be targeted, depending upon the route of administration and the site of administration. For example, a tissue which is closest to the site of injection in the direction of blood flow can be transfected in the absence of any specific targeting. If lipid carriers are used, they can be modified to

techniques such as probing with an antibody which specifically recognizes the gene product or a reporter gene product present in the expression cassette.

Typically, the therapeutic cassette is not integrated into the patient's genome. If necessary, the treatment can be repeated on an *ad hoc* basis depending upon the results achieved. If the treatment is repeated, the patient can be monitored to ensure that there is no adverse immune or other response to the treatment.

The invention also provides for methods for expanding a population of pancreatic β-cells *in vitro*. Upon isolation of the pancreas from a suitable donor, cells are isolated and grown *in vitro*. The cells which are employed are obtained from tissue samples from mammalian donors including human cadavers, porcine fetuses or another suitable source of pancreatic cells. If human cells are used, when possible the cells are major histocompatability matched with the recipient. Purification of the cells can be accomplished by gradient separation after enzymatic (e.g., collagenase) digestion of the isolated pancreas. The purified cells are grown in media containing sufficient nutrients to allow for survival of the cells as well as a sufficient amount of a β-cell proliferation inducing composition containing a gastrin/CCK receptor ligand and EGF receptor ligand, to allow for formation of insulin secreting pancreatic β cells. According to the invention, following stimulation the insulin secreting pancreatic β cells can be directly expanded in culture prior to being transplanted into a patient in need thereof, or can be transplanted directly following treatment with β-cell proliferation inducing composition.

Methods of transplantation include transplanting insulin secreting pancreatic β-cells obtained into a patient in need thereof in combination with immunosuppressive agents, such as cyclosporin. The insulin producing cells also can be encapsulated in a semi-permeable membrane prior to transplantation. Such membranes permit insulin secretion from the encapsulated cells while protecting the cells from immune attack. The number of cells to be transplanted is estimated to be between 10,000 and 20,000 insulin producing β cells per kg of the patient. Repeated transplants may be required as necessary to maintain an effective therapeutic number of insulin secreting cells. The transplant recipient can also, according to the invention, be provided with a sufficient amount of a gastrin/CCK receptor ligand and an EGF receptor ligand to induce proliferation of the transplanted insulin secreting β cells.

The following materials and methods were used in the studies reported by the working examples set forth below except as otherwise noted.

Animals. Mice, FVB and CD strain, were obtained from Taconic Farms, Inc., Germantown, NY. The TGF-α transgenic line MT-42 used, which expresses high levels of TGF-α from a metallothionein promoter, is described in Jhappan *et al*, *Cell*, *61*:1137-1146 (1990). Normal Wistar and Zucker rats were allowed normal chow *ad libidum* with free access to water and were acclimatized for one week prior to initiation of each study. Freshly prepared streptozotocin at a dose of 80 mg/kg body weight was administered by I.V. five to seven days after induction of diabetes, the rats were randomly allocated into groups for subsequent treatment. Hormones, TGF-α and rat gastrin were reconstituted in sterile normal saline containing 0.1% BSA. According to the predetermined treatment schedule for different studies, each animal received a single, daily i.p. injection of either TGF-α or gastrin alone (4.0 μg/kg body weight) or as a 1:1 (w/w) combination (total 8.0 μg/kg) or PBS for a period of 10 days.

INSGAS Transgene Construct. A *PvuII-RsaI* fragment encompassing nucleotides -370 to +38 of the rat insulin I gene (Cordell, B.G. *et al*, *Cell*, *18*:533-543 (1979)) was ligated into pGem1 (Promega Corp., Madison, WI). A 4.4 kb *Bam*H1-*Eco*R1 fragment containing 1.5 kb introns 1 and 2 and exons 2 and 3 of the human gastrin gene which encodes the preprogastrin peptide precursor was isolated and subcloned downstream of the rat insulin I fragment in pGem1 (Promega). The fragment is described in Wiborg, O., *Proc. Natl. Acad. Sci. USA*, *81*:1067-1069 (1984) and Ito, R., *et al Proc. Natl. Acada. Sci. (USA)*, *81*:4662-4666 (1984). The insulin promoter-preprogastrin INSGAS transgene construct was excised as a 4.8 kb *Xba*I-*Eco*R1 fragment.

Generation and Characterization of Transgenic Mice. The fragment, made as described above was prepared for microinjection as follows. It was isolated by agarose gel electrophoresis, purified by CsC1 gradient purification, and dialyzed extensively against injection buffer (5mM NaC1; 01. MM EDTA; 5mM Tris-HC1 pH 7.4). Fertilized oocytes from FVB inbred mice

binding assay (Bio-Rad Laboratories, Hercules, CA). Aliquots from the pancreata were tested in duplicate in a TGF-α radioimmunoassay, which measured competition with ^{125}I TGF-α for binding to a solid-phase rabbit antibody raised against the C-terminus of rat TGF-α (kit from BioTope, Seattle, WA).

Blood Glucose. Blood glucose was determined either after overnight fasting or after IPGTT by glucose oxidase method.

Tissue Insulin Analysis. At the end of each study, the animals were sacrificed and pancreas removed. Small biopsies were taken from separate representative sites throughout the pancreas and immediately snap-frozen in liquid nitrogen for immunohistochemistry, protein, and insulin determinations. Snap-frozen pancreatic samples (n = 5) were rapidly thawed, disrupted ultrasonically in deionized water and aliquots taken for protein determination and the homogenate subjected to acid/ethanol extraction prior to insulin determination by RIA.

Histological Analysis. The pancreata were removed, weighed, similarly oriented in cassettes, fixed in Bouin's solution and embedded in paraffin by conventional procedures.

Tissue Preparation and Immunohistochemistry. Freshly excised pancreata were dissected, cleared of fat and lymph nodes, fixed in Bouin's fixative, and then embedded in paraffin for sectioning. Routine sections were stained with hematoxylin and eosin according to standard methods. Pancreatic tissue from adult 17 week old MT-TGF-α (MT-42) transgenic mice were immunostained for insulin to examine the effect of TGF-α over-expression on islet development. Insulin positive cells in TGF-α-induced metaplastic ductules were identified using immunoperoxidase staining guinea pig anti-human insulin sera (Linco, Eureka, MO); a pre-immune guinea pig serum was used as a control. Immunohistochemistry was performed on 5μ paraffin sections by the peroxidase/antiperoxidase method of Sternberger using a monoclonal rabbit antigastrin antibody. *See*, Sternberger, L.A., *Immunocytochemistry*, 2nd Ed. (1979) NY: Wiley. 104-170.

quantitated by point counting morphometrics was 2.14 mg +/- 0.84 (mean +/- se, n = 5) in the TGF-α transgenic pancreas compared to 1.93 mg +/- 0.46 (n = 6) non transgenic litter mates.

Thus, TGF-α over-expression alone did not effect transition of these protodifferentiated duct cells into fully differentiated islets. This implies that islet differentiation requires other factors absent from the adult pancreas of TGF-α transgenic mice. Since differentiation of protodifferentiated islet precursors occurs during late fetal development, factors regulating this transition would likely be expressed in islets during this period. Among the factors expressed in the developing islets are the gastrointestinal peptides, the gastrins.

EXAMPLE 2

Pancreatic Gastrin Expression from the INSGAS Transgene

To examine the possible role of gastrin in regulating islet differentiation, transgenic mice were created that express a chimeric insulin promoter-gastrin (INSGAS) transgene in which the insulin promoter directs pancreas specific expression of the gastrin transgene (Figure 2A). Unlike the gastrin gene, insulin gene expression is not switched off after birth. Thus, the INSGAS transgene results in a persistence of gastrin expression in the adult pancreas.

The INSGAS transgene comprised 370 bp of 5' flanking DNA and the first non-coding exon of the rat insulin I gene. Cordell, B., *et al*, *Cell 18:533-543 (1979)*. It was ligated to a *Bam*H1-*Eco*R1 fragment containing 1.5 kb intron 1 and exons 2 and 3 of the human gastrin gene which encodes the preprogastin peptide precursor. Wiborg, O., *et al*, *Proc. Natl. Acad. Sci. USA, 81*:1067-1069 (1984); and Ito *et al Proc. Natl. Acad. Sci. USA, 81*:4662-4666 (1984). A 4.8 kb INSGAS fragment was isolated and microinjected into inbred FVB, one cell mouse embryos. Hogan, B. *et al*, *Manipulating the mouse embroy*: *A laboratory manual*, (1986) NY:Cold Spring Harbor.

Gastrin immunoreactivity in pancreatic and stomach extracts from transgenic and non-transgenic mice was assayed by radioimmunoassay using antisera 2604 (Rehfeld, J., *et al*,

and TGF-α, three groups of mice were bred with equivalent FVB/CD1 strain genetic backgrounds: non-transgenic control, TGF-α single transgenic and INSGAS/TGF-α double transgenics. All three groups of mice were placed on 50mM $ZnCl_2$ at 3 weeks of age. At 17 weeks of age, the animals were sacrificed and the pancreas removed for histological evaluation. The pancreas from TGF-α and INSGAS/TGF-α mice had similar gross morphological appearances: resilient, firm and compact in contrast to the soft diffuse control pancreas. TGF-α expression was equivalent in TGF-α and INSGAS/TGF-α groups when measured by Northern blot analysis (data not shown) and by radioimmunoassay. The pancreatic TGF-α immunoreactive peptide levels were 12.2 +/- 1 and 18.9 +/- 8ng/mg protein (Mean +/- SD) in the TGF-α and INSGAS/TGF-α mice, respectively.

Light micrographs of hematoxylin stained paraffin sections of pancreas from the three groups of mice studied (A: INSGAS/TGF-α; B: FVB/CD1 controls; and C: TGF-α) were made. The INSGAS/TGF-α pancreas had some areas of increased ductular complexes and slightly increased interstitial cellularity; the field shown (Figure 3A) had the most severely abnormal morphology seen in the five animals; most of the pancreas was indistinguishable from controls (Figure 3B). In contrast, the field of TGF-α pancreas (Figure 3C) was typical and showed the interstitial cellularity and fibrosis combined with florid ductular metaplasia described by Jhappan *et al*, *supra*.

Pancreatic gastrin synergistically interacts with TGF-α to increase islet mass and inhibit the ductular metaplasia induced by TGF-α over-expression. Mating the homozygous MT-TGF-α (MT-42) mice (TGF-α) with heterozygotic INSGAS mice gave offspring that were either heterozygotic TGF-α single transgenic or double transgenic containing both INSGAS and TGF-α transgenes (INSGAS/TGF-α). Since INSGAS were FVB strain and TGF-α were CD1 strain, TGF-α homozygotes and CD1 controls (CON) were both mated with FVB to produce FVB/CD1 strain background for all three groups of mice. Mice were treated with 50mM $ZnCl_2$ from 3 weeks until sacrifice at age 17 weeks. The pancreas was removed, weighed, similarly oriented in cassettes, fixed in Bouin's solution and embedded in paraffin. One random section from each animal was used to quantitate the relative volumes of ductules and islets by point-counting morphometrics (Weibel, E.R., *Lab Investig.*, *12*:131-155 (1963)). At least 2000 points over tissue were counted as intercepts of a 50 point grid at 170x

Group III: TGF-α + Gastrin: a combination of the above preparations was administered i.p. at the dose levels given above for 10 days.

Group IV: Control animals received an i.p. injection of vehicle alone for 10 days.

At the end of the study period (10 days), all animals were sacrificed and samples of pancreas taken as follows: five biopsy specimens (1-2 mg) of pancreatic tissue were taken from separate representative sites in each rat pancreas and immediately snap frozen in liquid nitrogen for analysis of insulin content. For analysis of pancreatic insulin content, the snap frozen pancreatic samples were rapidly thawed, disrupted ultrasonically in distilled water and aliquots taken for protein determination and acid/ethanol extraction prior to insulin radioimmunoassay (Green *et al*, (1983) *Diabetes 32*:685-690). Pancreatic insulin content values were corrected according to protein content and finally expressed as μg insulin/mg pancreatic protein. All values calculated as mean +/- SEM and statistical significance evaluated using Student's 2-sample *t*-test.

Table 1

Treatment of Normal Rats with TGF-α and Gastrin

Treatment	**Pancreatic Insulin Content** (μg insulin/mg protein)
Control	20.6+/-6.0
TGF-α	30.4+/-7.4*
Gastrin	51.4+/-14.0**
TGF-α + Gastrin	60.6+/-8.7***

* TGF-α vs. control, $p = 0.34$;
** gastrin vs. control, $p = 0.11$;
*** combination of TGF-α and gastrin, $p = 0.007$.

As shown in Table 1, above, pancreatic insulin content was significantly increased (p = 0.007) in the TGF-α + gastrin treated animals as compared to control animals; there was an

Table 2

Treatment of Streptozotocin Rats with TGF-α and Gastrin

Treatment	Pancreatic Insulin Content (μg Insulin/mg protein)
Control (STZ alone)	6.06+/-2.1
STZ plus TGF-α + Gastrin	26.7+/-8.9

The induction of diabetes by STZ was successful and produced a moderate but sustained degree of hyperglycemia. Total insulinopaenia was not sought so as to ensure that the study animals retained a functioning, but reduced β-cell mass.

As shown in Table 2, above, the pancreatic insulin content of the control streptozotocin treated animals was less than one third that of normal rats (20.6 ± 6.0 mg insulin/mg protein, *see* Table 1 above) as a result of destruction of β-cells by the STZ. In STZ animals treated with a combination of TGF-α and gastrin, the pancreatic insulin content was more than four-fold that of the animals which received STZ alone, and statistically the same as that of normal rats.

Diabetes mellitus is a disease in which the underlying physiological defect is a deficiency of β-cells as a result either of destruction of the β-cells due to auto-immune processes or of exhaustion of the potential for the β-cells to divide due to chronic stimulation from high circulating levels of glucose. The latter eventually leads to a situation when the process of β-cell renewal and/or replacement is compromised to the extent that there is an overall loss of β-cells and a concomitant decrease in the insulin content of the pancreas. The above results demonstrate that a combination of TGF-α and gastrin can be used to treat diabetes by stimulating the production of mature β-cells to restore the insulin content of the pancreas to non-diabetic levels.

EXAMPLE 6

Effects of TGF-α and Gastrin on IPGTT in STZ-Induced Diabetic Animals

Two groups (average body weight 103g) of STZ induced diabetic Wistar rats (n = 6/group) were treated for 10 days with a daily i.p. injection of either a combination of TGF-α and gastrin or PBS. Fasting blood glucose was determined for all rats on days 0, 6, and 10. In

gastrin was without effect on normal weight gain. Within error limits body weight gain was identical in all the groups.

The effect of TGF-α + gastrin treatment on fasting blood glucose in the obese Zucker rats was compared to the corresponding PBS controls. Fasting blood glucose was first significantly increased by day 15 (4.0 ± 0.6 vs. 5.0 ± 0.2) and this time point was chosen as the starting time for the 10-day treatment period with TGF-α + gastrin or with PBS control. Fasting blood glucose levels were not significantly altered by the TGF-α + gastrin treatment or by PBS. Fasting blood glucose values were lower in lean, as compared to obese animals whether or not they were treated with the growth factors or with PBS.

The results of treatment with TGF-$_\alpha$ and gastrin in the Zucker rat model of Type 2 diabetes showed no significant differences in blood glucose levels between the treatment and control groups, probably reflecting the transient hypoglycemic effect following a prolonged period (18 hrs) of fasting. The immunohistochemical studies revealed significant increases in the number of single foci of insulin containing cells in the TGF-$_\alpha$ and gastrin treated animals. as compared to control animals. These findings demonstrated an increase in single β-cells in adult rat pancreas following treatment with TGF-$_\alpha$ and gastrin. Interestingly, such single β-cell foci are not commonly seen in adult (unstimulated) rat pancreas. These findings support a therapeutic role for TGF-$_\alpha$ and gastrin in Type 1 and Type 2 diabetes since treatment is targeted at both β-cell neogenesis and replication.

The present invention is based in part on studies which demonstrated numerous insulin staining cells in the TGF-$_\alpha$-induced metaplastic ductules. The low level of exocrine and endocrine gene expression in the metaplastic ductal cells resembled that of protodifferentiated ductal cells seen in the early stage of fetal pancreatic development. Formation of islets (neogenesis) results from proliferation and differentiation of these protodifferentiated insulin expressing cells. Histologically this is manifested as islets appearing to bud from the pancreatic ducts (nesidioblastosis). In the MT-42 TGF-$_\alpha$ transgenic mice, ductular metaplasia was not seen in the immediate post-natal period, but only at 4 weeks of age. This indicates that TGF-$_\alpha$ over-expression induced insulin expression in duct epithelia rather than prolonging the persistence of islet precursors found in fetal pancreatic ducts. Although the metaplastic ductules contained numerous insulin positive cells, the islet mass of the TGF-$_\alpha$ transgenic mice was not increased over controls. The studies reported above demonstrate that complete islet cell neogenesis is reactivated *in vivo* in mammals in the ductular epithelium of the adult pancreas by stimulation with a gastrin/CCK receptor ligand, such as gastrin, and/or an EGF receptor ligand, such as TGF-$_\alpha$. Studies are reported on the transgenic over-expression of TGF-$_\alpha$ and gastrin in the pancreas which elucidate the role of pancreatic gastrin expression in islet development and indicate that TGF-$_\alpha$ and gastrin each play a role in regulating islet development. Thus, regenerative differentiation of residual pluripotent pancreatic ductal cells into mature insulin-secreting cells is a viable method for the treatment

What is claimed is:

1. A method for treating diabetes mellitus in an individual in need thereof, said method comprising:

administering to said individual a composition providing at least one receptor ligand selected from the group consisting of a gastrin/CCK receptor ligand and an EGF receptor ligand in an amount sufficient to effect differentiation of pancreatic islet precursor cells to mature insulin-secreting cells.

2. The method according to Claim 1, wherein said at least one receptor ligand is an EGF receptor ligand selected from the group consisting of EGF1-53, EGF1-48, or its EGF1-47 or EGF1-49 congener.

3. The method according to Claim 2, wherein said EGF1-53, EGF1-48, or its EGF1-47 or EGF1-49 congener is human EGF1-53, EGF1-48, or its EGF1-47 or EGF1-49 or its congener.

4. A method for providing a patient with diabetes in need thereof with a population of mature insulin-secreting beta cells, said method comprising:

transplanting into said patient cultured pancreatic islets which have been provided with a sufficient amount of at least one receptor ligand selected from the group consisting of a gastrin/CCK receptor ligand and an epidermal growth factor receptor ligand to induce proliferation of mature insulin-secreting beta cells of said islets prior to said transplanting.

5. The method according to Claim 4, wherein said diabetes is Type 2 diabetes.

6. The method according to Claim 4, wherein said gastrin/CCK receptor ligand is a gastrin.

14. A method for stimulating pancreatic islet cell neogenesis in an individual in need thereof, said method comprising:

administering to said individual a composition comprising at least one receptor ligand selected from the group consisting of a gastrin/CCK receptor ligand and an EGF receptor ligand in an amount sufficient to effect differentiation of pancreatic islet precursor cells to mature insulin-secreting islet cells, wherein said composition is administered systemically.

15. The method according to Claim 14, wherein said individual.

16. The method according to Claim 14, wherein both said gastrin/CCK receptor ligand and said EGF receptor ligand are administered.

17. The method according to Claim 16, wherein at least one of said gastrin/CCK receptor ligand and said EGF receptor ligand is a proteinaceous receptor ligand.

18. A method for treating diabetes mellitus in an individual in need thereof which comprises administering to the individual a composition providing a gastrin/CCK receptor ligand and an EGF receptor ligand in an amount sufficient to effect differentiation of pancreatic islet precursor cells to mature insulin-secreting cells.

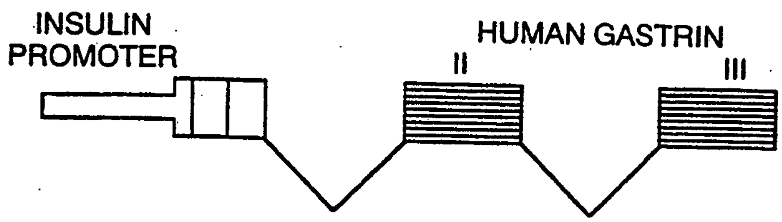
INSULIN
PROMOTER
HUMAN GASTRIN
II
III

FIG. 2A

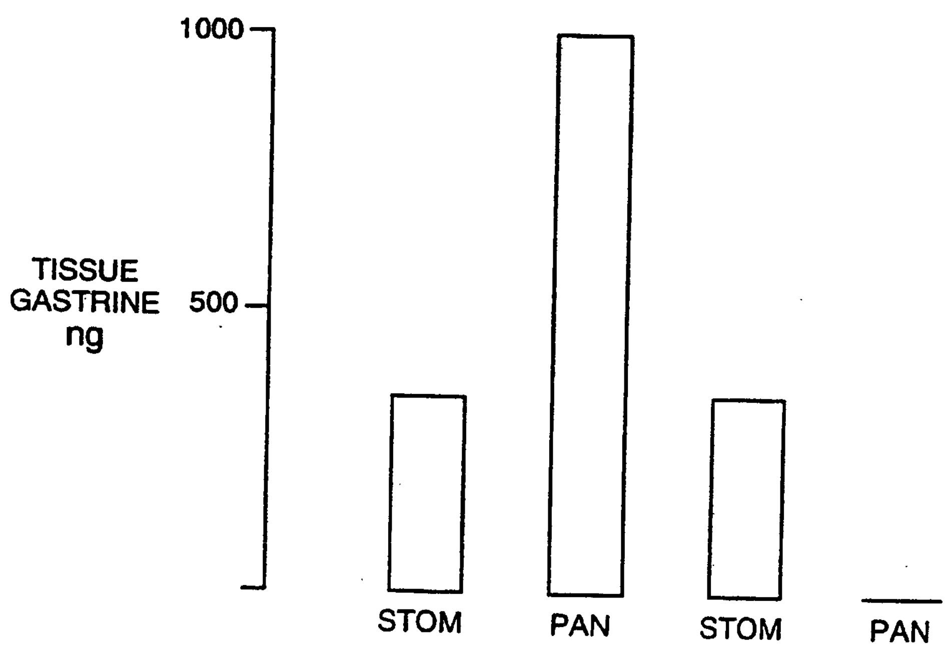
1000
500
TISSUE
GASTRINE
ng
STOM
PAN
STOM
PAN

FIG. 2B

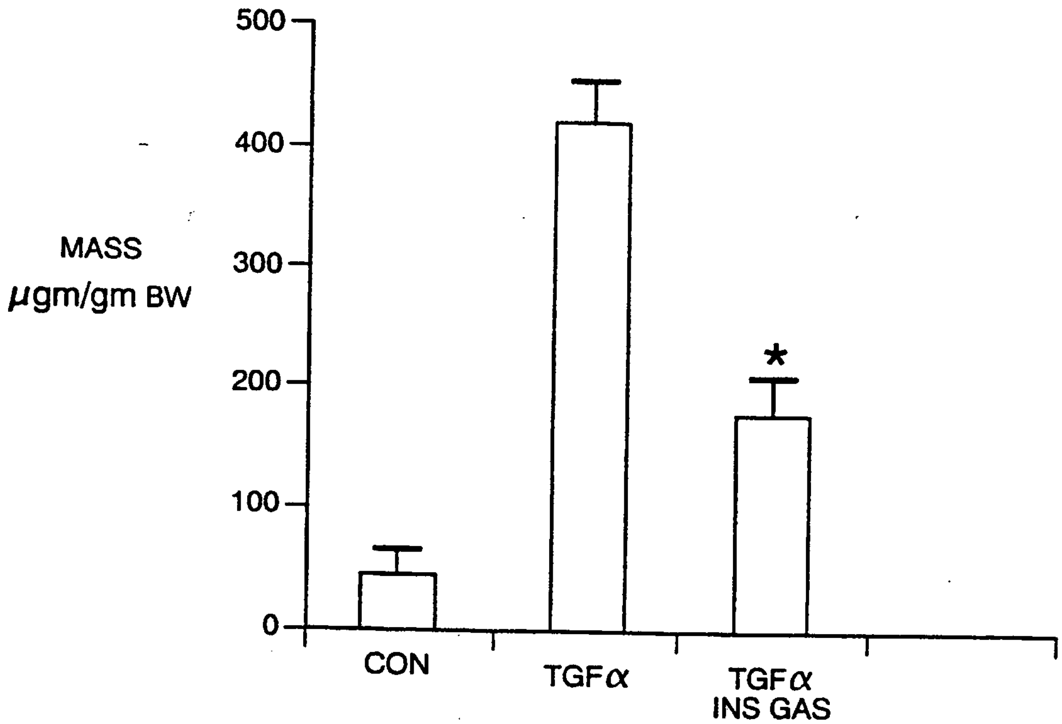

FIG. 4A

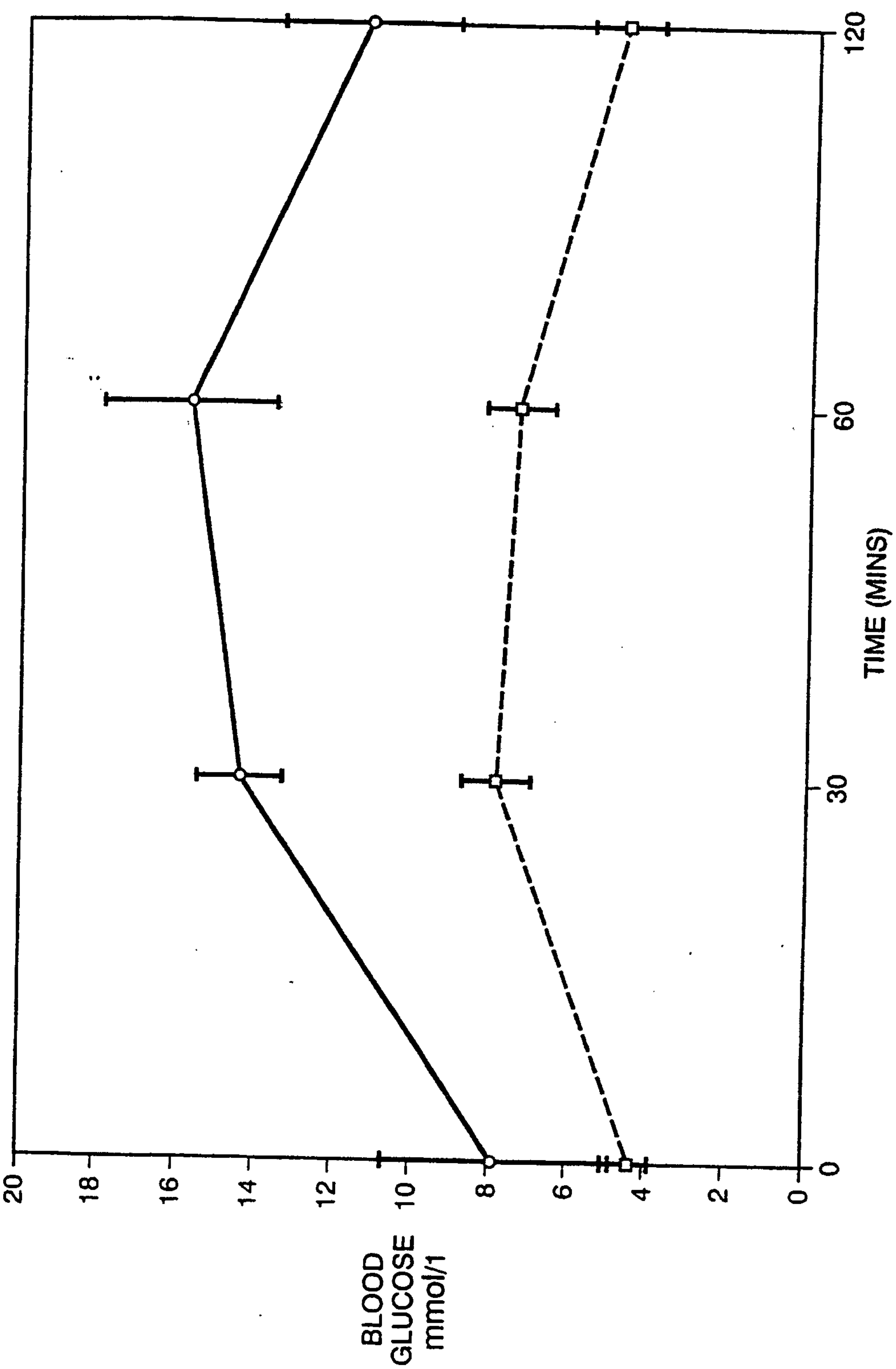
BLOOD GLUCOSE mmol/1
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS)
0
30
60
120

FIG. 5

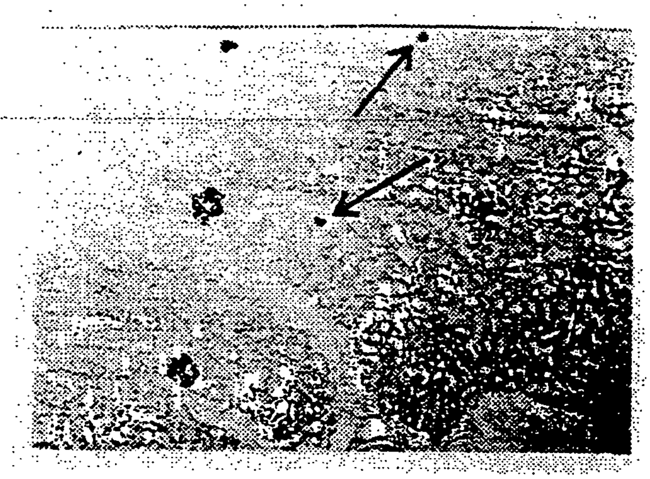

FIG. 7A

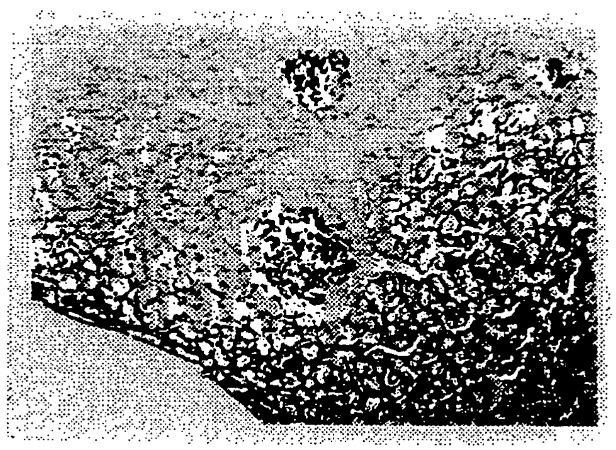

FIG. 7B

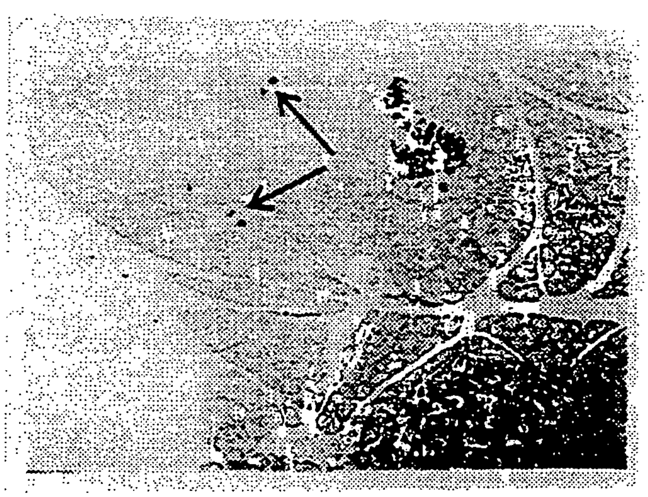

FIG. 7C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25463

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/22 A61K35/39 A61P5/48 //(A61K38/22,38:18)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 19785 A (RES TRIANGLE PHARM LTD) 27 July 1995 (1995-07-27) page 4, line 1 -page 5, line 2 page 9, line 4 -page 13, line 15; claims 1-20	1-18
A	WANG ET AL: "Function and Regulation of Gastrin in Transgenic Mice: A Review" YALE JOURNAL OF BIOLOGY AND MEDICINE,US,NEW HAVEN, CT, vol. 65, 1 January 1992 (1992-01-01), pages 705-713, XP002093891 the whole document	1-18

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2000	09/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niemann, F

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/25463

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-7,8 (in so far it concernes in vivo method),10-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

PATENT COOPERATION TREATY

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The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20 Switzerland

PATENT & TRADEMARK DEPT

PCT

NOTIFICATION OF DATE OF RECEIPT OF PRIORITY DOCUMENT OR OF PRIORITY APPLICATION NUMBER

(PCT Administrative Instructions, Section 323(a), (b) and (c))

Applicant's or agent's file reference ~~T01236-4-WO~~ 101336-0002-WO	Date of mailing *(day/month/year)* 24 September 2004 (24-09-2004)
International application No. PCT/CA2004/000648	International filing date *(day/month/year)* 30 April 2004 (30-04-2004)
Applicant WARATAH PHARMACEUTICALS, INC. ET AL	

1. [X] This receiving Office hereby gives notice of the receipt of the priority document(s) identified below on:

14 SEPTEMBER 2004.

2. [ ] This receiving Office hereby gives notice of the receipt of a request (made under Rule 17.1(b)) to prepare and transmit to the International Bureau the priority document(s) identified below on:

______.

Identification of the priority document(s):

Priority date	Priority application No.	Country or regional Office or PCT receiving Office
30 April 2003 (30-04-2003)	60/509,068	US

Name and mailing address of the receiving Office Commissioner of Patents Canadian Receiving Office Box PCT, Ottawa/Gatineau K1A 0C9 Facsimile No. (819) 953-9538	Authorized Officer Lucille Léonard (819) 953-1737